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ELECTRICAL PROPERTIES OF INDIVIDUAL CELLS ISOLATED FROM ADULT RAT VENTRICULAR MYOCARDIUM

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SUMMARY

1. Individual cells were isolated from adult rat ventricular myocardium by a collagenase digestion procedure.

2. Steady membrane potentials recorded with conventional intracellular glass micro-electrodes from cells in a modified Krebs solution containing 3·8 mM-KCl and 0·5 mM-$CaCl_2$ were less negative than -40 mV in most cells ($-25{\cdot}3 \pm 10{\cdot}9$ mV, mean ± S.D., 211 cells).

3. After addition of the potassium selective ionophore valinomycin (60 nM) to the bathing solution all recorded membrane potentials were more negative than -60 mV ($-74{\cdot}8 \pm 7{\cdot}0$ mV, sixty-three cells).

4. The internal concentration of potassium in the cells was determined as 120·8 ± 1·7 mM (± S.E., $n = 24$) by flame emission spectrometry after centrifugation through silicone oil, using tritiated water and D-[1-^{14}C] mannitol to estimate total and extracellular water in the pellet.

5. In the majority of cells in the standard solution the membrane potential recorded within a few msec of penetration was more negative than -70 mV ($-78{\cdot}4 \pm 9{\cdot}7$ mV, seventy-three cells). In sixty-six cells penetration initiated an action potential which overshot zero by $31{\cdot}3 \pm 7{\cdot}1$ mV. This overshoot was abolished by reducing the external sodium to 0·1 of the normal value, and reduced or abolished by addition of tetrodotoxin (30 μM).

6. Modifications of the standard bathing solution which increased the number of cells with steady recorded membrane potentials more negative than -60 mV were: isosmotic substitution of sucrose for NaCl; replacement of NaCl and KCl by sodium isethionate and potassium methyl sulphate; addition of 5 or 10 mM-$CaCl_2$; addition of 10 mM-$MnCl_2$.

7. For cells in solution containing 2·5 or 5·5 mM-$CaCl_2$, input resistances estimated from the amplitude of hyperpolarizations evoked by 200 msec current pulses were approximately 40 MΩ at a resting potential close to -80 mV and became much greater as cells were depolarized. Time constants measured at the resting potential were approximately 8 msec.

8. In certain experimental conditions, repeated spontaneous action potentials were recorded from contracting cells, and in quiescent cells evoked action potentials

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could be initiated by applying brief depolarizing pulses through the micro-electrode. Action potentials were coincident with contractions.

9. It is concluded that the resting potential of these isolated cells is normally more negative than -70 mV, and that the cells retain the ionic mechanisms necessary for the generation of active currents.

INTRODUCTION

The intricate anatomy of the myocardium, coupled with distinct morphology and function of specialized component cells, often presents acute problems in the interpretation of experimental data obtained from the whole organ. One experimental approach to minimize some of the difficulties encountered is the use of single cardiac cells isolated enzymically from adult mammalian myocardium and a technique has been developed in this laboratory whereby tissue dissociation is achieved by *in vitro* organ perfusion with crude bacterial collagenase (Gould & Powell, 1972; Powell & Twist, 1976*a*). Myocytes isolated in this manner from adult rat heart have typical superficial and subcellular morphology (Gould & Powell, 1972; Powell, Steen, Twist & Woolf, 1978*a*), show respiratory coupling (Powell & Twist, 1975, 1976*a*) and have retained responsiveness to β-adrenergic stimulation (Powell & Twist, 1976*b*; Powell, Terrar & Twist, 1978*c*). To date, however, there has been no systematic study of the electrical activity of these isolated cells, although such data are central to both the evaluation of the preparation as a useful experimental model and also to gain an insight into the electrophysiology of cardiac cells uncoupled physically from their usual syncitial state.

Experiments are presented to show that under suitable incubation conditions membrane potentials which are comparable to those reported for multicellular preparations can be recorded from isolated cells using intracellular micro-electrodes. It is also demonstrated that these cells can generate conventional action potentials, indicating that the relevant ionic mechanisms have been preserved during the isolation procedures.

Some of these results have appeared previously in abstract form (Powell, Terrar & Twist, 1978*b*, *c*).

METHODS

Cell preparation. Although the general procedures for isolating and purifying cells from adult rat heart have been outlined (Gould & Powell, 1972; Powell & Twist, 1976*a*) there has been no detailed account of the experimental protocol. Experience has shown that there are several critical points to observe in order to achieve routine preparations of isolated cells having the electrical characteristics reported here, so a complete description of the technique is presented.

The animals used were female Sprague–Dawley rats approximately 12 weeks old and 250 g in weight, fed *ad libitum* on a standard chow diet. Hearts were perfused retrogradely through the aorta at 37 °C on the apparatus shown in Fig. 1, which incorporated a heart chamber (*B*1), reservoir (*B*2) and perfusion column (*A*) of standard design (Baker, 1951). The perfusion system was primed with 50 ml. perfusion fluid, a nominally Ca-free Krebs–Ringer bicarbonate buffer of the following composition: NaCl 118·5 mM, $NaHCO_3$ 14·5 mM, KCl 2·6 mM, KH_2PO_4 1·18 mM, $MgSO_4$ 1·18 mM, glucose 11·1 mM. Bovine albumin (essentially fatty acid free, prepared from their Fraction V albumin by Sigma Chemical Co., Poole, Dorset) was added to give a final concentration of 1 mg/ml. and the medium gassed with a mixture of O_2 (95%) and CO_2 (5%)

to maintain the pH at 7·40 ± 0·05. Coronary flow rate was monitored by the simple method of timing the drops descending from the apex of the heart, 1 drop per second corresponding to a flow of approximately 5 ml./min.

A rat was killed by stunning and cervical dislocation, the heart removed quickly and placed in ice-cold bicarbonate buffer of the same composition as the perfusion fluid until beating ceased, then transferred to a tared beaker also containing cold buffer and weighed. The heart was then mounted on the glass cannula (*C*, Fig. 1) of the perfusion apparatus, perfused retrogradely through the aorta (Langendorff) at a pressure of 70 mmHg until clear of blood and the perfusate

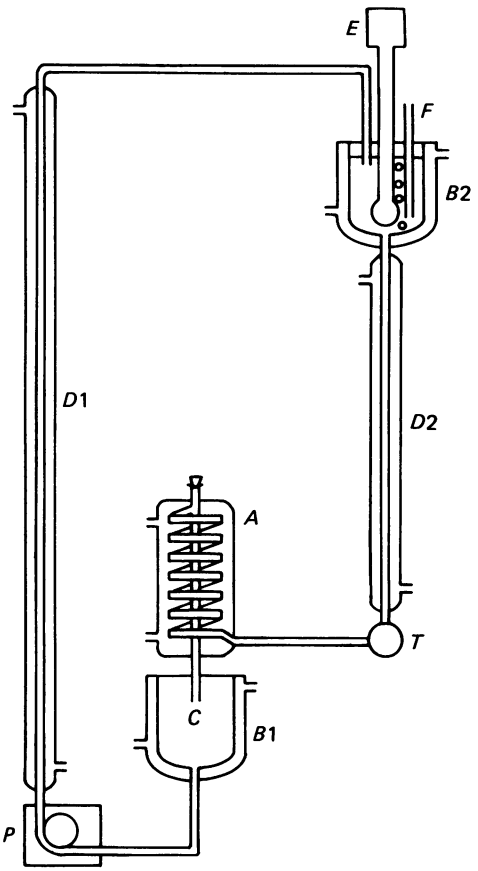

Fig. 1. The perfusion apparatus. Reservoirs (*B*1, *B*2) and perfusion column (*A*) are of standard design (Baker, 1951). Jacketed tubes *D*1 (90 cm long) and *D*2 (70 cm long) are of 2 mm i.d. and the remaining connecting tubes are siliconized and 2 mm i.d. Paristaltic pump *P* recirculates fluid, which is gassed by line *F* while pH is monitored by probe *E*. Flow rate is adjusted manually via tap *T*.

used for this initial washing was collected. The remaining buffer was recirculated at a coronary flow rate of 2 drops per second for 4 min, during which time a volume of Krebs–Ringer solution equal to that of the discarded washing solution and containing an additional 25 μM-$CaCl_2$ was added to 20 mg of crude bacterial collagenase (Type 1, *Cl. histolyticum*, Worthington Biochemical Corporation, Freehold, New Jersey). This enzyme solution (approximately 10 ml.) was added to reservoir *B*2 at the end of the equilibration period and the flow rate adjusted to 1 drop per sec. Perfusion was continued for a period of 16·5 min per g heart wet weight and throughout this time coronary flow was maintained constant by manual adjustment of perfusion pressure via tap *T*. At the end of the perfusion period the heart was reweighed, the atria together with any

connective tissue were trimmed away and the ventricles cut from base to apex into four pieces, which were then chopped transversely into 1 mm thick slices by a mechanical tissue chopper (McIlwain, The Mickle Laboratory Engineering Company, Godalming, Surrey). Following this, the tissue was placed in a poly-tetrafluoroethylene beaker containing 20 ml. gassed perfusate to which had been added additional fatty acid free albumin (10 mg/ml.). Incubation of the tissue slices continued at 36 °C until dispersion was achieved by gentle agitation through a 10 ml. serological pipette. After this time, generally 10–15 min, the resulting cell suspension was filtered through 250 μm mesh nylon gauze and centrifuged at ambient temperature for 1 min at 22 g, then washed once by resuspension in 20 ml. albumin-free buffer followed by centrifugation.

The washed cell pellets were resuspended in a total of 20 ml. albumin-free buffer and then layered in 10 ml. aliquots each on 40 ml. Krebs–Ringer solution containing bovine albumin 40 mg/ml. (Pentex Fraction V, Miles Laboratories Limited, Stoke Poges, Bucks.) in precision-walled glass tubes 15 cm long and 2·1 cm internal diameter. These tubes were centrifuged for 70 sec at 22 g, the supernatants aspirated and the cell pellets resuspended in albumin-free buffer, layered again on 40 ml. albumin solutions and centrifuged at the same speed for 65 sec. The final cell pellets were resuspended in a total volume of 20 ml. Krebs–Ringer solution containing albumin 20 mg/ml. (fraction V) and 0·5 mM-$CaCl_2$, and 10 ml. aliquots of suspension were stored at 37 °C in stoppered 25 ml. conical flasks. These suspensions were gassed at regular intervals with a 95% O_2, 5% CO_2 gas mixture.

Cells obtained in suspension were of two distinct types morphologically, rod-shaped cells approximately 100 μm long and 20 μm wide with visible cross-striations, which by their exclusion of the stains trypan blue and lissamine green, together with their superficial and subcellular ultrastructure, were considered to be intact myocytes, and round cells with no organized structure which were contracted myocytes damaged by the isolation procedure (Gould & Powell, 1972; Powell & Twist, 1976*a*; Powell *et al.* 1978*a*). On the order of 70% of cells in suspension were initially of the intact form (Powell & Twist, 1976*a*) and it is this cell type which is the subject of the present study.

The isolation procedure was evolved empirically but as described does routinely provide a preparation of isolated cells in suspension. All chemicals were Analar grade or equivalent and glassware coming into contact with cells was siliconized (Repelcote, Hopkin and Williams, Chadwell Heath, Essex). Double glass-distilled water was used throughout and every new batch of collagenase, fatty acid free and fraction V albumin was tested to ensure continuity of cell yield and purity. During the past 6 yr, only one batch of enzyme has proved unsuitable and it has been found convenient to purchase the collagenase in multiples of 1 g lots, since storage at 4 °C over long periods does not retard efficacy.

Electrophysiology. Membrane potentials were recorded with conventional glass micro-electrodes filled with 3 M-KCl and having resistances in the range 30–90 MΩ (measured with 1 nA of current in the bathing solution). In the majority of experiments the potential difference between micro-electrode and bath electrode was recorded differentially using a system employing FET amplifiers (Burr–Brown 3522 K) for the input stage (Terrar, 1978). A Neurolog NL 102 DC preamplifier was used in those experiments where current was to be injected into the cell via the recording micro-electrode since this system incorporates a bridge network which is designed to cancel any voltage drop occurring in the micro-electrode as a consequence of the injected current. The 10–90% rise time of both recording systems could be adjusted using capacity compensation to 300 μsec. The injected current was measured from the voltage drop across a 100 kΩ resistor (using a Burr–Brown 3670 instrumentation amplifier) in the reference line to the Neurolog preamplifier; an independent measure of current could be made from the monitor output of this preamplifier with the system balanced before impaling a cell, in which case similar results were obtained without the 100 kΩ resistor in the reference line. Voltage and current data were displayed on Tektronix 565 and Gould Advance OS 4000 oscilloscopes and recorded on a Racal Store 4 FM tape recorder (15/16 in. sec^{-1} to 15 in. sec^{-1}, 1 db down at 5 kHz for the fastest speed). The majority of experiments were made on 1 ml. samples of cell suspension in a Perspex bath mounted on a heated (37 °C) microscope stage. The cells settled to the bottom of the bath where they could be impaled with micro-electrodes. All measurements were made during the first 30 min after removal from the prepared stock of cells and during this period there was no systematic change in the recorded membrane potentials with time. In a few experiments cells

were mounted in the surface of an agar island in a continuous stream of flowing solution (37 °C, bubbled with 95% O_2, 5% CO_2), the composition of which could be changed while keeping a recording micro-electrode in place in a cell (Powell *et al.* 1978*c*). The procedure for mounting the cells in this way was to place 200 μl. 2% (w/v) agar in Krebs–Ringer at 45 °C (at which temperature it was liquid) in the perspex bath, add 30 μl. cell suspension and start superfusion with 37 °C Krebs–Ringer, causing the agar to rapidly cool to 37 °C (at which temperature it formed a smooth gel) thus trapping the cells; this procedure took less than 10 sec. Stable membrane potentials could be recorded from single cells in these conditions for at least 3 hr.

Solutions. The 'standard' solution had the same composition as the perfusion fluid but was supplemented with 0·5 mM-$CaCl_2$. In some experiments 2, 5 or 10 mM-$CaCl_2$ or 10 mM-$MnCl_2$ was added to this solution. The 10 mM-$MnCl_2$ and 10·5 mM-$CaCl_2$ solutions were made up immediately before use, since some precipitation occurred in these media after long periods. In low-Na solution all the NaCl was replaced by an osmotically equivalent amount of sucrose (210 mM). In low Cl-solution, NaCl was replaced by Na isethionate (Sigma) and KCl was replaced by K methyl sulphate (Hopkins & Williams). In experiments where the K^+ concentration was to be increased this was done either by substitution of KCl for NaCl or by addition of K methyl sulphate to the standard solution (see text). Valinomycin and tetrodotoxin were obtained from Sigma Chemical Co., Poole, Dorset.

Determination of intracellular potassium concentration. Incubations were performed in quadruplicate in 2 ml. conical tubes under a 95% O_2 5% CO_2 atmosphere at 37 °C. To 100 μl. cell suspension was added 10 μl. tritiated water (150 μc/ml.) and 10 μl. D-[1 − ^{14}C]mannitol (20 μc/ml., both obtained from The Radiochemical Centre, Amersham, Bucks.) and the tube gassed, capped and incubated for a period of 30 min. At the appropriate time, 100 μl. suspension was layered on 100 μl. oil (Versilube, Alpha laboratories, Greenford, Middx.) beneath which was 100 μl. 0·5 M-perchloric acid in 33 mM-HCl, the whole mixture being made up in a plastic microfuge tube. The tube was centrifuged at 10,000 *g* for 1 min (152 Microfuge, Beckman Instruments Inc. Palo Alto, California) and 50 μl. supernatant added to 4·95 ml. 3 mM-NaCl in a plastic cup and stored at room temperature before determination of K^+ concentration. A further 20 μl. supernatant was added to 180 μl. perchloric acid/HCl mixture in a microfuge tube, which was centrifuged for 1 min at 10,000 *g* and 50 μl. of the deproteinized supernatant transferred to 2 ml. scintillation fluid for counting. The remainder of the supernatant on the oil in the microfuge tube was discarded and the tube held with approximately 75% of its length immersed in a solid CO_2/acetone mixture for 25 s, then cut through the oil layer (Mackie, Simpson, Mee, Tait & Tait, 1977). After the rest of the oil had been aspirated, the tube was thawed and a 50 μl. aliquot of the contents taken for scintillation counting and the remainder transferred into 1 ml. NaCl in a plastic cup using a glass capillary drawn from a siliconised pasteur pipette. The cut tube base was washed twice with 100 μl. 3 mM-NaCl and the washings also placed in the plastic cup, 10 μl. concentrated HNO_3 added, the cup capped and stored overnight at room temperature.

^{14}C and ^{3}H radioactivity in the medium and the cell extracts were measured in a liquid scintillation counter (Tri-Carb Model 3380, Packard Instruments, Caversham, Berks.) and count rates corrected for background and channels overlap. The total water (tritium) space and extracellular ([^{14}C]mannitol) space were calculated for each cell pellet and the difference between these values used as an estimate of intracellular water. K^+ concentrations in the cell extract and diluted medium were determined with an SP 90A series 2 Atomic Absorption Spectrometer in emission mode at 766·5 nm wave-length (Pye-Unicam Instruments Ltd., Cambridge). The total K^+ content of the original cell pellet was corrected for the K^+ contribution from the trapped extracellular fluid and this value used with the water space measurement to calculate intracellular K^+ concentration. Recoveries of added radioactivity and K^+ using these extraction procedures were not determined in the present experiments but have been shown to be complete when similar techniques were used on isolated adrenal cortical cells (Mendelsohn & Warren, 1975; Mackie *et al.* 1977).

RESULTS

Fig. 2*A* and *C* shows records of the potential differences obtained relative to the bath as individual cells were penetrated by the micro-electrode. The final steady potentials were approximately −25 mV for cells incubated in the standard solution containing 0·5 mM-$CaCl_2$. When penetrations were recorded at high speed (Fig. 2*B* and *D*) it was clear that potentials were much more negative than −25 mV during the first few msec after impalement, but there was a decay of the recorded voltage to

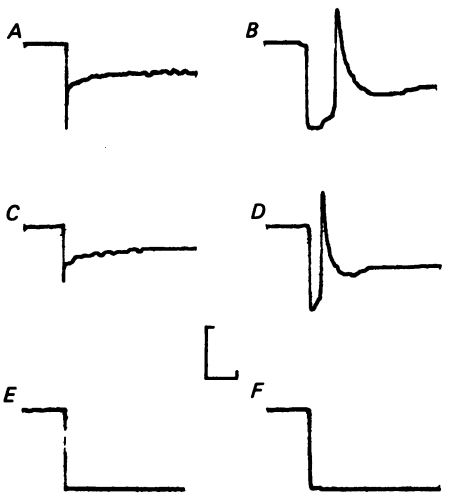

Fig. 2. Impalements of individual ventricular cells. Records show potential difference between bath electrode and micro-electrode as cells were impaled (occurring at one quarter of the sweep) either at slow sweep speed (*A*, *C*, *E*, calibration 1 sec) or at fast sweep speed (*B*, *D*, *F*, calibration 10 msec). Two cells were in standard solution (*A*, *B*; *C*, *D*) and one in standard solution + 60 nM-valinomycin (*E* and *F*). Voltage calibration, 50 mV. Records from FM tape recorder (15 in sec^{-1}) to digital storage oscilloscope, then at slow speed (approximately 55 sec sweep) to pen recorder; a similar technique was used for Figs. 6, 7 and 14.

less negative values thus triggering an action potential which did not follow a normal repolarization pattern but resulted in stabilization of the recorded potential at a level less negative than the initial value. This sequence of events will be discussed in more detail later, but it may be postulated at this point that the amplitude of the negative voltage transient can be used as an estimate of the initial resting membrane potential of the cells. The contrast between initial and steady values for membrane potential is evident from a comparison of the distribution of 211 steady potentials (Fig. 3*A* and Table 1) with the distribution of seventy-three initial potentials (Fig. 3*B* and Table 2). It might of course be postulated that the initial potential is artifactual and that although the steady potentials are much less negative than those recorded in intact myocardial tissue (Weidmann, 1956; Noble, 1975; Coraboeuf, 1978) it is not possible to record more negative potentials from these isolated cells using the preparative and recording techniques employed. To test this hypothesis, potentials were recorded in the presence of 60 nM-valinomycin, a potent K ionophore, which should drive the cell membrane potential towards the K equilibrium potential (Pressman, 1976). Under these conditions, records of cell penetration show an

abrupt transition to values of about −75 mV (Fig. 2*E* and *F*) and this potential is maintained throughout the record. The distribution of steady membrane potentials in cells exposed to valinomycin is illustrated in Fig. 3*C* and mean values quoted in Table 1.

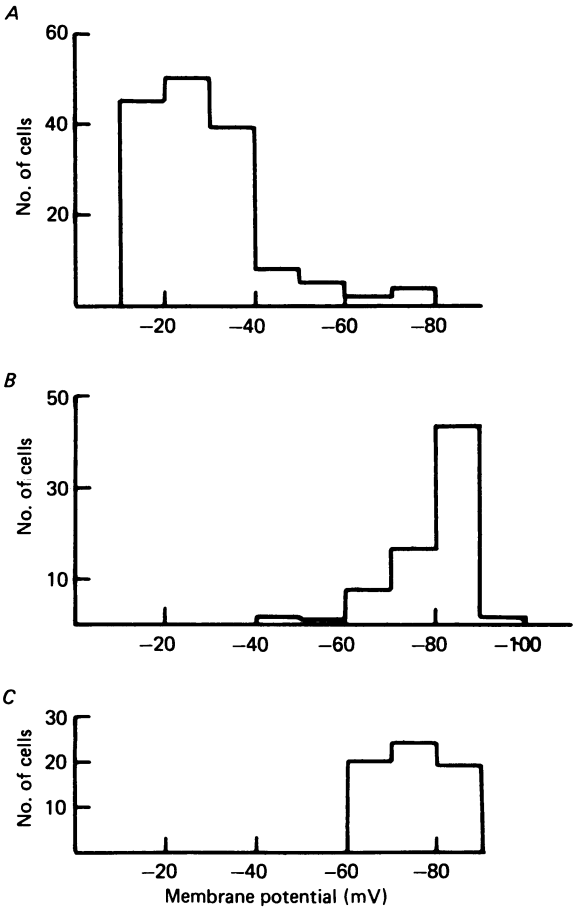

Fig. 3. Distributions of membrane potentials of ventricular cells. *A*, steady potentials in standard solution; *B*, initial potentials in standard solution; *C*, steady potentials in standard solution + 60 nM-valinomycin

TABLE 1. Steady membrane potentials recorded with an intracellular micro-electrode from ventricular cells in a variety of solutions

Solution	Membrane potential mean ± S.D. (mV)	No. of cells
Standard (0·5 mM-$CaCl_2$)	−25·3 ± 10·9	211
Standard + 60 nM-valinomycin	−74·8 ± 7·0	63
Low chloride (0·5 mM-$CaCl_2$)	−49·8 ± 24·6	106
Low NaCl	−41·9 ± 19·5	47
42 mM-KCl	−26·3 ± 2·7	23
2·5 mM-$CaCl_2$	−33·2 ± 13·2	49
5·5 mM-$CaCl_2$	−86·6 ± 6·6*	34
10·5 mM-$CaCl_2$	−86·8 ± 5·1*	20

* Means from cells which survived to 'switch' to more negative membrane potentials

More negative steady potentials can be recorded in solutions having low $[Cl^-]_o$ or reduced NaCl

The more negative potentials recorded with valinomycin and also estimated from the high speed traces suggest that after impalement the cells adopt a steady membrane potential which is much more positive than the K equilibrium potential. Since cellular chloride movements have a stabilizing effect on potential changes initiated

TABLE 2. Initial membrane potentials recorded immediately after insertion of the micro-electrode into ventricular cells in a variety of solutions

Solution	Membrane potential mean ± S.D. (mV)	No. of cells
Standard (0·5 mM-$CaCl_2$)	−78·4 ± 9·7	73
Standard + 60 nM-valinomycin	−75·6 ± 1·1	9
Low chloride (0·5 mM-$CaCl_2$)	−81·8 ± 6·8	14
Low NaCl	−69·4 ± 9·2	12
Standard + 30 μM-tetrodotoxin	−75·9 ± 12·7	29
42 mM-KCl	−32·5 ± 4·4	10
2·5 mM-$CaCl_2$	−80·6 ± 7·5	31
5·5 mM-$CaCl_2$	−79·4 ± 4·6	25
10·5 mM-$CaCl_2$	−75·3 ± 9·6	21

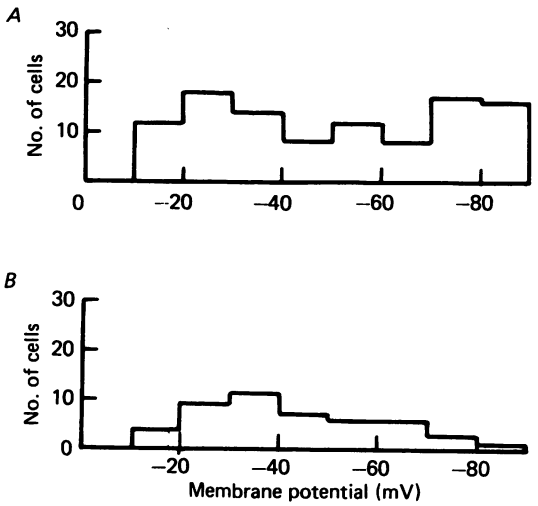

Fig. 4. Distributions of steady membrane potentials of ventricular cells. *A*, low Cl^- solution; *B*, low NaCl solution.

by alterations in other components of membrane current (Hodgkin & Horowicz, 1959; Adrian, 1960; Carmeliet, 1961; Wiggins & Cranefield, 1976) it is possible that removal of this anion might result in cell potentials assuming more negative values. The distribution of 106 potentials in low Cl^- solutions, with isethionate and methylsulphate as replacement anions, is shown in Fig. 4*A* and it is seen that 39% of the recorded steady potentials were more negative than −60 mV, compared to only 3% of 211 potentials in normal Cl^- solutions (cf. Fig. 3*A*).

At a membrane potential of −25 mV and assuming a high internal K^+ concentra-

tion ($[K^+]_i$) any K^+ current will be outward and a balancing inward current must be carried by an ion with an equilibrium potential positive to this value. One likely candidate is Na^+ and in solutions where $[Na^+]_o$ has been reduced to 10% of the normal level by substitution of NaCl with an osmotically equivalent amount of sucrose, 24% of forty-seven potentials were more negative than −60 mV (Fig. 4*B*). It is not possible, however, to attribute these results solely to the low $[Na^+]_o$, since both Cl^- and Na^+ were reduced in these experiments.

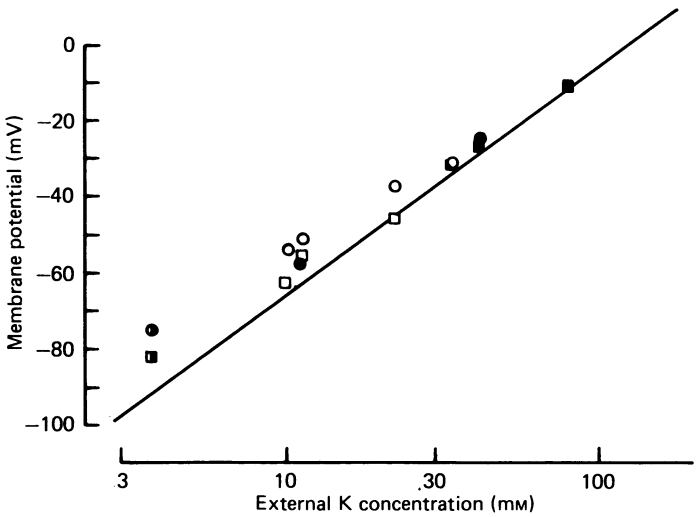

Fig. 5. Steady membrane potentials of ventricular cells in the presence of 60 nM-valinomycin plotted as a function of the concentration of K in the external solution (log scale). Points represent means of two to sixty-three potentials in normal Cl^- (circles) or low Cl^- solution (squares). K concentration was increased from the standard level of 3·8 mM (◑, ■) either by adding K methyl sulphate (open symbols) or by substituting the K salt for the Na salt (filled symbols; KCl for NaCl in normal Cl^-, K methyl sulphate for Na isethionate in low Cl^- solution).

There is a large spread in both distributions of potentials, with values ranging from potentials similar to those found for the normal bathing solution (Fig. 3*A*) to more negative values comparable with the mean value for potentials recorded when 60 nM-valinomycin is added (Table 1 and Fig. 3*C*).

Isolated cells have a high $[K^+]_i$

The values of recorded potential more negative than −60 mV obtained in the valinomycin and ion-substitution experiments suggest that in at least some of the isolated cells there has not been a substantial reduction in their internal potassium concentration. To examine this point further, $[K^+]_i$ was measured directly using the techniques outlined in Methods. In three complete experiments a mean value for $[K^+]_i$ of 120·8 ± 1·7 mM (± S.E.; n = 24) was obtained and similar results were found in three other experiments for which complete quadruplicate data sets were not available. If this is a representative value for $[K^+]_i$ and the assumption is correct that potentials measured with valinomycin present reflect the relevant potassium

equilibrium potential, then a semilogarithmic plot of recorded potentials with ionophore against $[K^+]_o$ should obey the Nernst equation, with a slope of 61·5 mV/decade at 37 °C and a $[K^+]_o$ intercept at zero membrane potential numerically equal to $[K^+]_i$, assuming equal activity coefficients for K^+ in both cytoplasm and external solution. A series of experiments were then carried out in both normal and low $[Cl^-]_o$ solutions where $[K^+]_o$ was varied in the range 3·8–80 mM by either adding K methyl

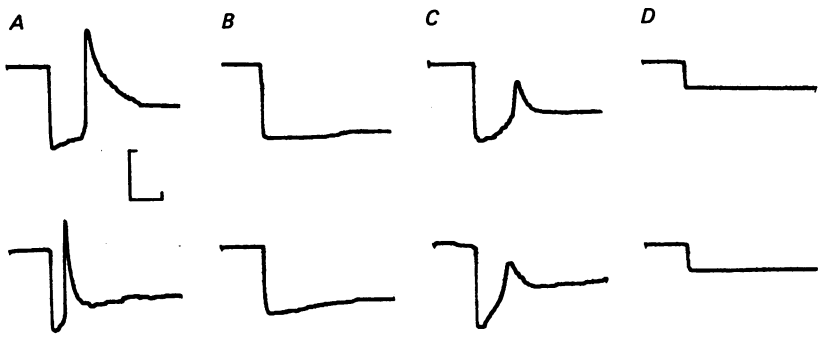

Fig. 6. Records of impalements of eight ventricular cells. *A*, in standard solution; *B*, in low NaCl solution; *C*, in standard solution + 30 μM-tetrodotoxin; *D*, in solution containing 42 mM-KCl. Calibrations: 50 mV and 10 msec.

sulphate directly to the bath or substituting KCl iso-osmotically for NaCl. These data are shown in Fig. 5 where it can be seen that a Nernst plot with a $[K^+]_i$ of 120 mM is in reasonable accord with the experimental results. A slightly better fit can be obtained by assuming that the ratio of intracellular to extracellular K^+ activities is about 0·8.

Solutions inhibiting action potential generation modify the initial voltage response to micro-electrode insertion

It was postulated above that in the majority of cells impaled in the standard solution, action potentials were triggered by insertion of the micro-electrode. These action potentials (Figs. 2*A* and 6*A*) overshot zero by approximately 30 mV (Table 3). If it is the case that impalement of the cell with a micro-electrode does disturb membrane steady-state conductances through time and voltage dependent changes to produce an action potential, then any interventions which inhibit action potential generation should attenuate or abolish this portion of the initial voltage response.

In Fig. 6*B* are shown tracings of the initial potential time course when cells were impaled in low-Na sucrose solution. The voltage spike following impalement was attenuated and the overshoot abolished (Table 3). When penetrations were made in solutions containing TTX (30 μM) reduced spike potentials were also recorded (Fig. 6*C* and Table 3). These data are consistent with reduced availability of rapid inward Na current resulting in the cell being unable to generate a conventional action potential. A further test of this hypothesis is to examine initial potential records in solutions with high $[K^+]_o$, where membrane depolarization would also decrease Na availability. Fig. 6*D* shows that in solutions containing 42 mM-$[K^+]_o$

there was no action potential and the initial recorded potential was maintained at a remarkably steady level following impalement. The time course of this recording of membrane potential was similar to the results obtained in the valinomycin experiments (Fig. 3*E* and *F*) but the amplitude was less negative than both the initial potential in standard solution and the steady potential in 60 nM-valinomycin (3·8 mM-$[K^+]_o$).

TABLE 3. Amplitude of overshoot of action potentials initiated by insertion of the micro-electrode into ventricular cells in a variety of solutions. Means do not include failures to initiate overshooting action potentials which occurred in some conditions

Solution	Amplitude of overshoot mean ± S.D. (mV)	No. of action potentials	No. of failures
Standard (0·5 mM-$CaCl_2$)	31·3 ± 7·1	66	7
2·5 mM-$CaCl_2$	32·3 ± 7·1	31	0
5·5 mM-$CaCl_2$	34·3 ± 4·0	24	0
10·5 mM-$CaCl_2$	32·5 ± 8·1	21	0
Standard + 30 μM-tetrodotoxin	8·8	2	27
Low NaCl	—	0	12

It can be seen from Table 2 which summarizes data from a series of experiments that even though the sequence of potential changes following insertion of the micro-electrode could be modified by attenuating the triggered action potential, the amplitudes of the initial, most negative, potentials were similar in all solutions containing 3·8 mM-K.

Steady potentials more negative than −70 mV can be recorded in high $[Ca^{2+}]_o$ or $[Mn^{2+}]_o$ solutions

If the normal resting potentials of these isolated cells are more negative than −70 mV and micro-electrode penetration leads to the adoption of a new steady state at potentials of −20 to −30 mV, then are there conditions under which the initial potentials can be restored? Fig. 7*B* and *C* show records of a cell impalement carried out in a solution containing 10·5 mM-$[Ca^{2+}]_o$. The expected sequence of initial transient followed by an action potential (Table 3) and depolarization was observed, but there then followed, often after a few seconds, a transition to a second steady potential which was more negative than −70 mV. A similar sequence occurred in cells exposed to 10 mM-$MnCl_2$ (Fig. 7*D*) with the difference that hyperpolarization when Mn^{2+} was present was not accompanied by action potentials, which were initiated in high $[Ca^{2+}]_o$ solutions. Although some cells did not survive penetration in 10·5 mM-$[Ca^{2+}]_o$ (Fig. 7*A*), it can be seen from Table 1 that the steady potentials in those cells which did survive to 'switch' to a more negative potential were not only comparable to the initial potentials (Table 2) but were consistent with the valinomycin experiments. Similar values for steady potentials were obtained in 5·5 mM-$[Ca^{2+}]_o$ which was sufficient to support 'Switching' (Table 1).

Measurement of cell input resistance by current injection

Estimations of cell input resistances were made in solutions containing 5·5 mM-$CaCl_2$ for two main reasons. First, determination of input resistance during the transition phase to more negative potentials might clarify the mechanisms underlying this process. Secondly, the negative stable membrane potentials observed in this solution should facilitate comparison of input resistances of isolated cells with

Fig. 7. Records of impalements of ventricular cells in solutions containing 10·5 mM-$CaCl_2$ (*A*, *B*, *C*) or 10 mM-$MnCl_2$ (*D*). Some cells did not survive this procedure, for example, cell *A* lost its rod shape and membrane potential soon after the end of this record. Cells retaining their rod shape showed a rapid transition of the membrane potential from the initial steady level to a more negative potential (*B*, *C*, *D*). Action potentials which occurred immediately after penetration and during the transition to a more negative potential in 10·5 mM-$CaCl_2$ were not faithfully recorded because at this sweep speed the sampling rate of the digital storage oscilloscope was inadequate. Calibrations: 20 mV and 10 sec. A d.c. change (80 mV) in recorded potential was made towards the end of record *B*.

measurements of membrane resistances reported for multi-cellular preparations. Measurements of cell input resistances were made by injecting 200 msec hyperpolarizing current pulses (0·1–0·5 nA) into the cells and monitoring the voltage deflexions. Only one micro-electrode was used and a bridge circuit was employed to compensate for voltage drops in the electrode itself.

Fig. 8 illustrates experiments of this kind and it can be seen that following impale-

ment cell input resistance increased from its initial value while the membrane potential either changed marginally or became less negative. As the cell hyperpolarized to approximately -80 mV there was a marked reduction in measured input resistance. In ten cells, the ratio of maximum input resistance (which occurred at approximately -20 mV to -40 mV) to that measured at the final stable potential was 5·8 (mean, range 2·8–8·7). These results would not have been expected if the impaled

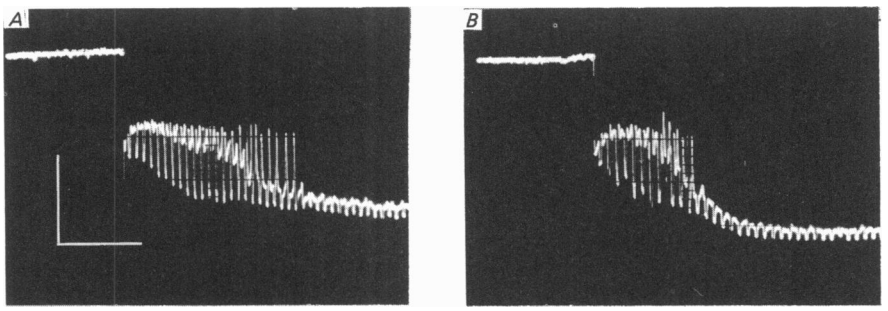

Fig. 8. Changes in estimated input resistance during transition of recorded membrane potential to a more negative level in solution containing 5·5 mM-$CaCl_2$. Two cells (*A* and *B*). Current pulses (200 msec, 0·11 nA) which were applied at 1 pulse sec^{-1} before and after impalement evoked hyperpolarizations of the impaled cells. Notice that during the transition to a more negative steady membrane potential the hyperpolarizations evoked by the constant current pulses became smaller. Action potentials which were not faithfully reproduced at this sweep speed (inadequate sampling rate) are apparent as upward deflexions. The break in record *B* indicates a change in tape speed. Calibrations: 50 mV and 10 sec.

cell could be modelled by a constant membrane resistance in parallel with a significant variable electrode shunt which became higher in resistance as Ca ions promoted sealing of the membrane around the electrode. For this model, hyperpolarization would have been accompanied by an increase in the measured input resistance. There are at least two possibilities which could account for the experimental findings: (i) the cells exhibit a marked non-linear current–voltage relationship so that the cell input resistance is much lower at -80 mV than at less negative membrane potentials, in which case any depolarization caused by an electrode shunt increases cell resistance and so exaggerates the effect of the shunt relative to that which would have occurred had the resistance remained at its initially low level, while a small amount of sealing around the electrode would promote hyperpolarization and a decrease in the measured input resistance; (ii) cell conductance could be increased due to an increase in membrane permeability to K ions brought about by calcium influx around the micro-electrode (Meech, 1974; Isenberg, 1975, 1977*a–c*) resulting in hyperpolarization.

To investigate the *I–V* profiles of these cells, steady currents of either polarity were injected to change the resting potential, while at the same time continuing the hyperpolarizing current pulses. It was found (Fig. 9) that as the cells were depolarized the voltage deflexions produced by the constant current pulses increased in amplitude to a maximum and then decreased at even larger depolarizations. These observations are clearly consistent with the postulate of a non-linear *I–V* curve,

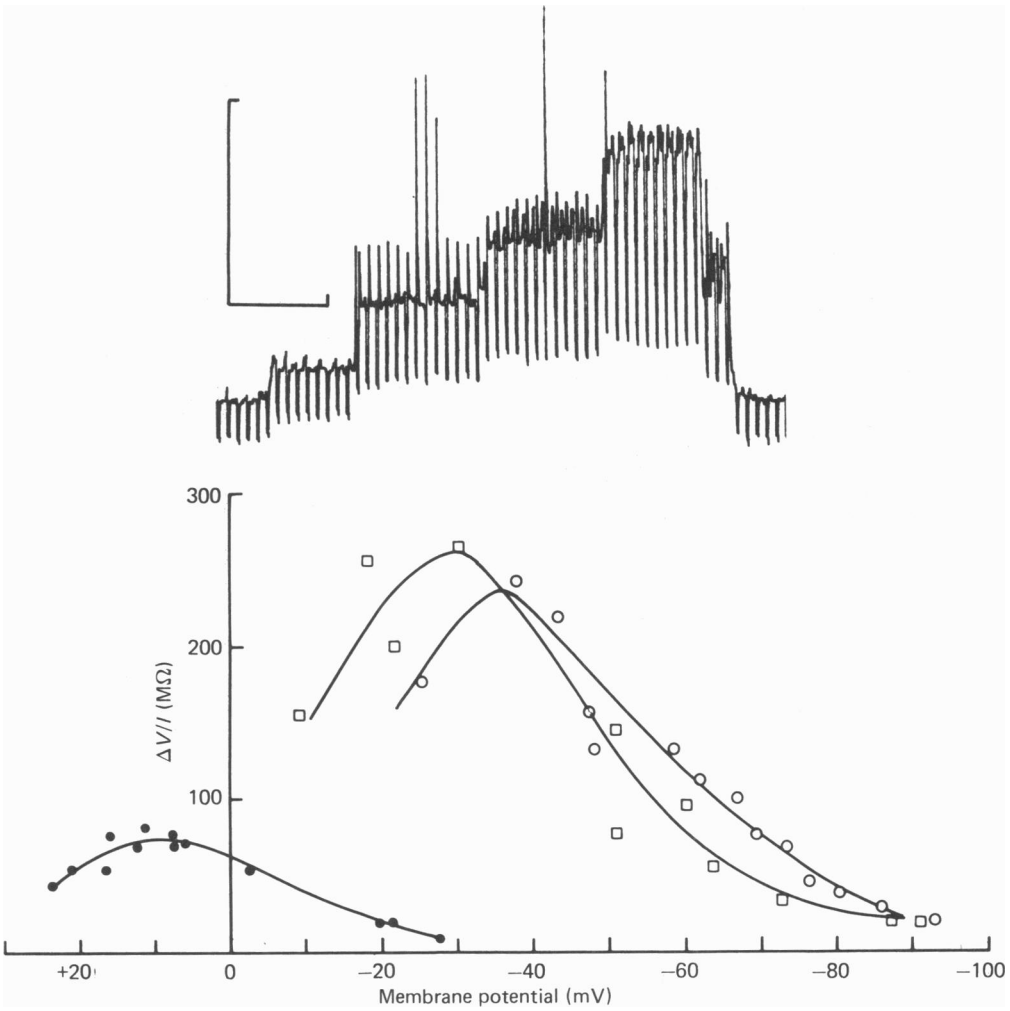

Fig. 9. Upper panel: records of hyperpolarizations (downward deflexions from steady membrane potential) evoked by constant current pulses (0·165 nA) as membrane potential was changed by DC current. Calibrations: 50 mV and 10 sec. Lower panel: estimated input resistance (change in membrane potential, ΔV (mV) divided by applied current, I (nA)) plotted as a function of membrane potential. Solutions contained: 2·5 mM-$CaCl_2$ (○, standard solution + 2 mM-$CaCl_2$); 5·5 mM-$CaCl_2$ (□, standard solution + 5 mM-$CaCl_2$); 42 mM-KCl (●, modified standard solution in which 38 mM-KCl replaced 38 mM-NaCl). Each series of points from a single cell.

although the precise form of the relation cannot be determined by this method, since the large voltage deflexions caused by the current at certain levels of depolarization (cf. Fig. 9) preclude estimation of the 'slope resistance' in that region. Increase in slope resistance during depolarization is a well known phenomenon in intact cardiac tissue and is accounted for by inward rectification of outward conductance (Beeler & Reuter, 1970; Noble, 1975.)

If the above interpretation of the experimental results is correct, the data provide a lower estimate of the shunt resistance around the micro-electrode in these conditions,

since if the impaled cell can be modelled by a shunt resistance in parallel with a variable cell input resistance then the shunt resistance must always be at least as large as the measured input resistance. In the cells illustrated in Fig. 8 the maximum measured input resistances were 311 and 340 MΩ.

Estimates of cell input resistances were also made for cells bathed in solutions containing differing $[Ca^{2+}]_o$. The relation between estimated input resistance and

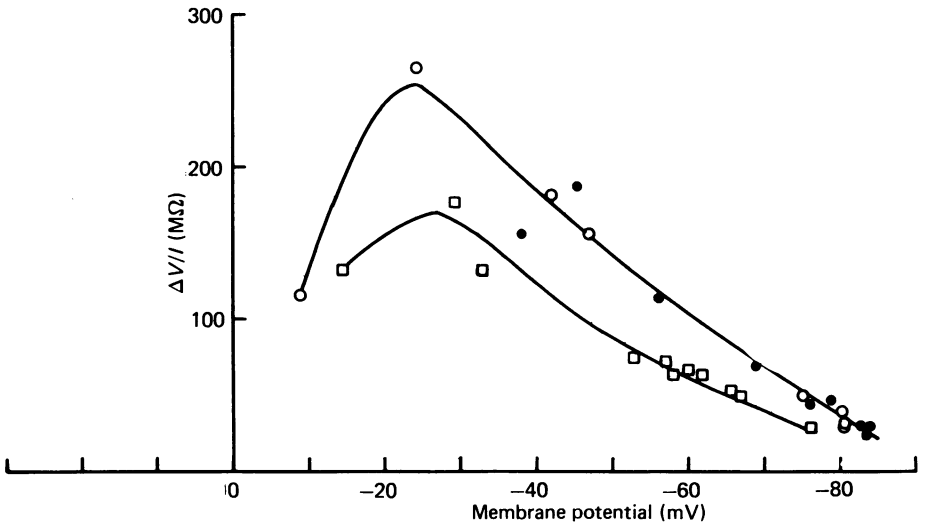

Fig. 10. Estimated input resistances ($\Delta V/I$ (MΩ)) plotted as a function of membrane potential. All records from a single cell in flowing solution measured in sequence 5·5 mM-$CaCl_2$ (●), 0·5 mM-$CaCl_2$ (□), and 5·5 mM $CaCl_2$ (○).

membrane potential was similar in solutions containing 2·5 mM-$CaCl_2$ to that found for 5·5 mM-$CaCl_2$ (Fig. 9). However, in five cells where $[Ca^{2+}]_o$ was 0·5 mM the mean input resistance of 26 ± 14 MΩ (± S.D.) for a membrane potential of −20 to −30 mV was lower than the values obtained in the higher calcium solutions at comparable potentials (cf. Fig. 9). Furthermore, the input resistance showed little change in 0·5 mM-$[Ca^{2+}]_o$ as the membrane potential was varied in the range of approximately +10 to −60 mV. These findings might reflect either a difference in the cell membrane characteristics in solutions containing 0·5 mM-$CaCl_2$ or the existence of a microelectrode shunt which contributed more to the measured input resistance in these conditions than when extracellular Ca concentration was raised.

To examine this point further, cells were impaled first in a solution containing 5·5 mM-$[Ca^{2+}]_o$ and the solution then changed to a Ca concentration of 0·5 mM using a superfusion system (see Methods). Experiments of this kind are illustrated in Fig. 10 where it can be seen that (i) as the membrane potential was varied the input resistance of cells in 0·5 mM-$[Ca^{2+}]_o$ did change in a manner which was qualitatively similar to that observed at higher concentrations, (ii) at approximately −80 mV the measured input resistances were comparable with those shown in Fig. 9 and (iii) at a membrane potential of −20 to −30 mV the estimated input resistances were higher than for cells maintained throughout impalement and recording in 0·5 mM-$[Ca^{2+}]_o$. These results are consistent with the hypothesis that there is an improve-

ment in the seal around the micro-electrode for cells impaled in the higher Ca^{2+} solutions, but it is impossible to rule out the possibility that once cells have 'switched' to a more negative membrane potential in these solutions there is a change in their membrane characteristics which is partially preserved even in 0·5 mM-Ca solutions.

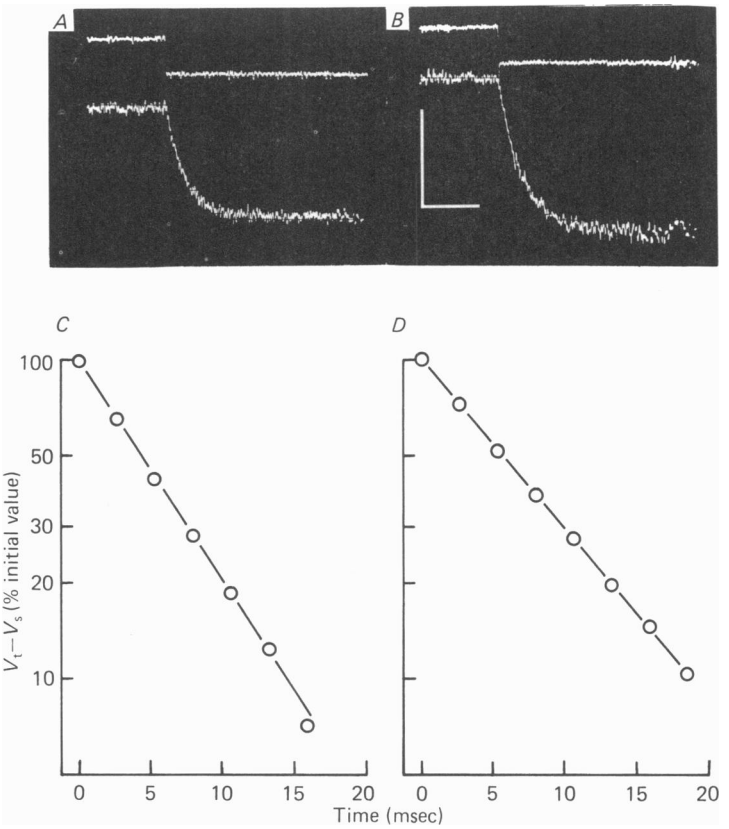

Fig. 11. Records of hyperpolarizations evoked by rectangular current pulses in two cells (*A* and *B*). Upper trace shows applied current (amplitude 0·165 nA); lower traces show hyperpolarizations. Calibrations: 5 mV and 20 msec. The lower panel (*C* and *D*) shows the data from *A* and *B* presented in the form of a semilogarithmic plot of $V_t - V_s$, where V_t is the membrane potential at time t and V_s is the steady level of membrane potential during the hyperpolarizing pulse, against time. $V_t - V_s$ is expressed as a percentage of its value at the beginning of the pulse. Time constants measured from these plots were 6·2 msec for cell *A* and 8·2 msec for cell *B*.

A few experiments were also carried out in the standard solution with 0·5 mM-Ca^{2+} concentration when $[K^+]_o$ was increased from 3·8 to 42 mM by substituting KCl isosmotically for NaCl. Fig. 9 shows an experiment of this kind in which it was found that the variation in estimated input resistance with membrane potential was similar in shape to that determined in 3·8 mM-$[K^+]_o$ (Figs. 9 and 10) but was displaced to more positive potentials.

Skeletal muscle fibres and Purkinje fibres also show displaced $I-V$ curves at increased extracellular K^+ concentrations (Adrian, 1969; Noble, 1975). The data in

Fig. 9 also suggest that in high potassium the maximum input resistance was reduced compared to that found in the normal solution, but this effect was not seen clearly in all of the cells examined.

Time constants of hyperpolarizations evoked by rectangular current pulses

The hyperpolarizations evoked by rectangular current pulses applied to cells at a stable resting potential more negative than -70 mV with the micro-electrode in place were exponential (Fig. 11*A–D*).

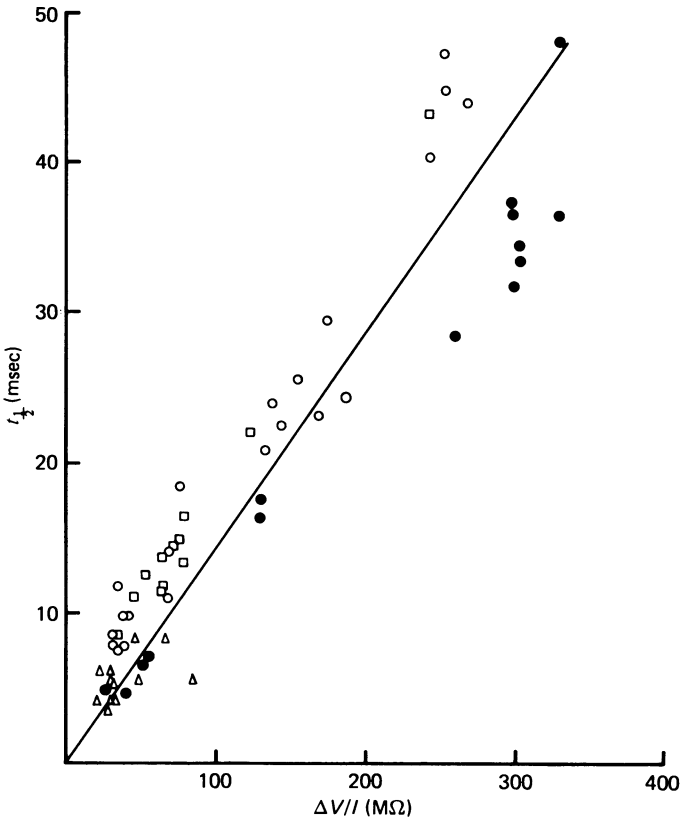

Fig. 12. Half-times ($t_{\frac{1}{2}}$ msec) of hyperpolarizations evoked by rectangular current pulses plotted as a function of estimated input resistance ($\Delta V/I$ (MΩ)). One cell in 5·5 mM-$CaCl_2$ (●). Second cell in 5·5 mM-$CaCl_2$ (□), 0·5 mM-$CaCl_2$ (○), and 5·5 mM-$CaCl_2$ (□) in sequence. Twelve other cells (△).

In twelve cells examined in this way (two in 2·5 mM-$CaCl_2$, ten in 5·5 mM-$CaCl_2$) the mean input resistance at the resting potential was 39 ± 19 MΩ ($\pm$ S.D.) and the mean time constant was $8{\cdot}1 \pm 2{\cdot}2$ msec. If the cell can be modelled by a parallel input resistance and input capacitance, and if during depolarization by steady current the estimated input resistance increases while the capacitance remains approximately constant, the time constant should increase with input resistance. Fig. 12 shows data from two experiments (one cell in 5·5 mM-$CaCl_2$, the other in 5·5, 0·5, and 5·5 mM-$CaCl_2$ in sequence) to test this hypothesis, together with values from the

twelve cells used to estimate mean time constant at the resting potential. Errors in these measurements were expected to increase as the input resistance increased because the large hyperpolarizations approximated less to an exponential and were more variable; consequently half-times were measured rather than time constants. Despite the increasing scatter of points at high estimated input resistances, it can be seen that there was an approximately linear increase in half-time with input resistance (Fig. 12).

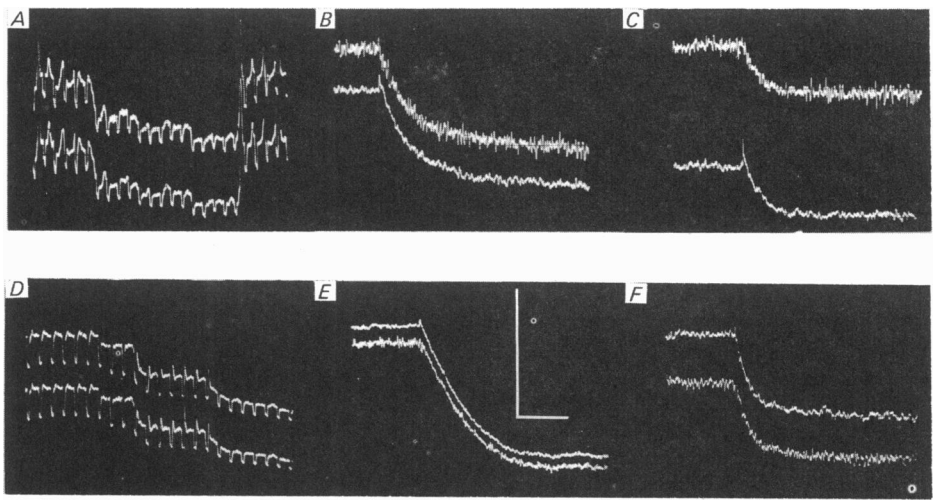

Fig. 13. Simultaneous records of membrane potential from two micro-electrodes at different sites in a single cell (electrode separation approximately 40 μm) are shown in each pair of traces. Records *A*, *B* and *C* from one cell (resting potential −68 mV); *D*, *E* and *F* from a second cell (resting potential −47 mV). Current pulses (200 msec, 0·165 nA) evoked hyperpolarizations in all records. The pairs of traces in *A* and *D* show parallel changes during application of steady currents to change the membrane potential. Records *B*, *C*, *E* and *F* show the hyperpolarizations evoked by current pulses at two membrane potentials in the traces shown in *A* and *D* at a faster time base and increased amplification. Calibrations: 40 mV and 4 sec (*A*), 100 mV and 4 sec (*D*), 10 mV and 20 msec (*B*, *C*, *F*) or 20 mV and 20 msec (*E*). Records from the electrode attached to the bridge circuit are lower traces in *A* to *C* and upper traces in *D* to *F*. The vertical separation between each pair of traces is for clarity and does not imply a difference in steady potential between the two recording sites.

Hyperpolarizations recorded simultaneously from two sites in a single cell

In some experiments two independent records of membrane potential during current injection were obtained by first impaling the cell with the current passing electrode* (attached to the bridge circuit as described above) and then inserting a second voltage-recording electrode 30–50 μm from the first, so that the electrodes were approximately one-third of a cell length from each other and from the ends of the cell. Records of experiments of this kind in two cells which survived this procedure in solution containing 5·5 mM-$CaCl_2$ are shown in Fig. 13. It can be seen that the two independent records of membrane potential showed parallel changes when steady current was injected (Fig. 13*A* and *D*), and that the amplitude and time course of hyperpolarizations evoked by current pulses were similar at the two recording

sites (Fig. 13*B*, *C*, *E* and *F*). Steady membrane potentials recorded at the two sites were in agreement to within 3 mV. Similar results were obtained in eight other cells.

Spontaneous and evoked action potentials recorded from individual ventricular cells

In the standard solution spontaneous action potentials were recorded from some of the cells with resting potentials more negative than −40 mV, and examples are shown in Fig. 14*A*, *B* and *C*. It can be seen that the shape of these action potentials

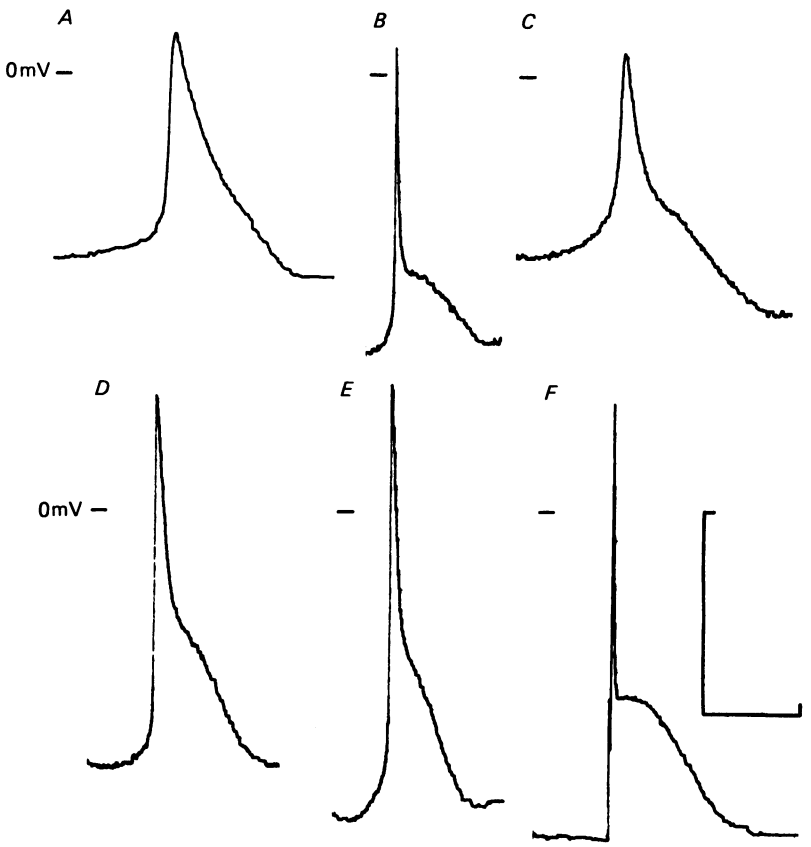

Fig. 14. Records of action potentials in individual cells. Either spontaneous (*A* to *E*) or evoked by depolarizing current pulses (*F*). Solutions contained 0·5 mM-$CaCl_2$ (*A*, *B*, *C*), 2·5 mM-$CaCl_2$ (*D*), or 5·5 mM-$CaCl_2$ (*E* and *F*). Calibrations: 50 mV and 200 msec.

was variable, the most common in these conditions being that of Fig. 14*A* and *C*. The action potential of Fig. 14*B* was recorded from the same cell as Fig. 14*C* and consequently the difference in shape of these two action potentials cannot reflect a difference in cell type; the difference may reflect the membrane potential from which the action potentials were initiated and detailed studies on this will be reported in a future publication. Spontaneous action potentials initiated from approximately −70 mV in solutions containing 2·5 and 5·5 mM-$CaCl_2$ are illustrated in Fig. 14*D*

and *E*. In all the above cells action potentials were accompanied by contractions monitored visually under the microscope. In cells which were electrically and mechanically quiescent after 'switching' to approximately −85 mV in solutions containing 5·5 mM and 10·5 mM-$CaCl_2$, evoked action potentials and contractions could be initiated by applying depolarizing current pulses; this is illustrated in Fig. 14*F* which shows an action potential from a cell to which 0·5 msec pulses were applied at 1 pulse sec^{-1} through the microelectrode to initiate the response. The difference in shape between action potentials in Fig. 14*E* and *F* may reflect the difference in the rate of firing (the spontaneous action potentials of Fig. 14*E* were occurring at approximately three to four action potentials per second in this case); further studies on this will also be reported in a future publication.

DISCUSSION

The major finding of the present investigation is that individual muscle cells isolated from adult rat ventricular myocardium can maintain resting potentials more negative than −70 mV and also retain the ionic mechanisms to support action potentials, despite the fact that they have been uncoupled physically from their usual syncitial state. Cell membrane potential can be estimated from the amplitude of the initial voltage transient recorded following impalement with a micro-electrode, but the recording of stable potentials more negative than −70 mV requires specific experimental conditions, perhaps the most convenient being the presence of 5·5 mM-$CaCl_2$ or greater. Once steady recordings at this level are achieved, then external Ca^{2+} concentrations can be reduced to 2·5 or 0·5 mM without substantial depolarization (Powell *et al.* 1978*c*). This experimental requirement for high extracellular Ca during electrode insertion may explain in part why Fabiato, Fabiato & Sonnenblick (1971) and Fabiato & Fabiato (1972) reported that although it was possible to record action potentials from intact single cells, these evolved from a low resting potential ranging from −30 to −50 mV and had little or no overshoots, since their recordings were carried out in solutions containing 0·05–1 mM-$CaCl_2$.

The value for intracellular $[K^+]_i$ reported here of 120 mM is about 20 % lower than that determined by Page & Page (1968) in rat left ventricular tissue. Part of the discrepancy could arise from the fact that sulphate was used by Page & Page as an extracellular marker, whereas mannitol was used in the present experiments. However, taking intracellular K^+ to be 120 mM, intracellular K^+ activity as 0·612 (Lee & Fozzard, 1975) and extracellular K^+ activity as 0·75 (Robinson & Stokes, 1965), the K^+ equilibrium potential would be about −87 mV for the isolated cells used in the present experiments when bathed in a solution containing 3·8 mM-KCl. Not only is this value consistent with the valinomycin experiments in which $[K^+]_o$ was varied (Fig. 5, where it was noted that a ratio of intracellular to extracellular K^+ activities of about 0·8 would provide a good fit with the experimental data) but it is also compatible with an estimate of −80 mV for cell resting potential (Table 2), since the cells would not be expected to behave as a perfect K^+-electrode, especially at a $[K^+]_o$ below about 5 mM (Weidmann, 1956; Noble, 1975).

At a resting membrane potential of −80 mV, an isolated cell has an input resistance of about 40 MΩ (Figs. 9, 10 and 12). If the interior of the cells were isopotential,

an assumption which is consistent with the data presented in Fig. 13, the specific membrane resistance (R_m, Ω cm^2) could be calculated by multiplying the input resistance by the membrane surface area. The irregular nature of the myocardial cell surface architecture makes precise determination of cell surface area difficult, but our own experiments indicate that many cells can be approximated by a rectangular block 100 μm $\times$ 20 μm $\times$ 6 μm, giving a surface area of approximately 6000 μm^2 and a specific membrane resistance of $(4 \times 10^7 \times 6 \times 10^{-5})\Omega$ cm^2 or 2·4 kΩ cm^2. Page & McCallister (1973) calculated from their stereological measurements that a 100 μm long rat ventricular cell would have a total plasma membrane area (external sarcolemma + T-system + transverse boundary) of about 8000 μm^2, which would make R_m 3·2 kΩ cm^2. These values, though approximate, are of the same order of magnitude as Weidmann's estimate of 9·1 $\pm$ 1·0 kΩ cm^2 for R_m in trabecular muscle (Weidmann, 1970).

Membrane time constants measured in these experiments of approximately 8 msec at a membrane potential of -80 mV are longer than those determined by Weidmann (1970) and correspond to a cell capacitance of 0·2 nF for an input resistance of 40 MΩ. Values for specific membrane capacitance calculated using the two estimates of cell membrane area mentioned above are 2·5 and 3·3 μF cm^{-2}; these values are also larger than Weidmann's estimate of 0·81 μF cm^{-2}, but are still of acceptable magnitude for biological tissues (Sakamoto, 1969; Beeler & Reuter, 1970).

Despite the approximate agreement between these measurements of electrical properties of isolated heart cells and corresponding values obtained in multicellular preparations, it remains possible that the enzyme treatment during the isolation procedure may have caused some modification of the electrical characteristics. Other evidence against a major modification of membrane characteristics during cell separation is provided by the experiments of Stauber, January & Schottelius (1977) who have reported that collagenase treatment of mammalian skeletal muscle had little or no effect on resting or action potentials, and Lee, Weeks, Kao, Akaike & Brown (1979), using isolated cells prepared by the techniques described here, have shown that Na current kinetics in isolated cells are similar to those reported for other preparations (Colatsky & Tsien, 1979).

Another argument that enzyme treatment has not caused substantial damage to the cells is the observation that they respond to adrenergic drugs (both to increase their content of cyclic adenosine 3′,5′-monophosphate (Powell & Twist, 1976*b*) and to modify the rate of firing and shape of action potentials recorded with intracellular electrodes (Powell *et al.* 1978*c*)), and therefore their receptors and subsequent metabolic steps must still be intact.

Furthermore, the stability and magnitude of the resting membrane potentials measured in the present study provide evidence against gross sarcolemmal damage and the presence of large non-specific membrane shunts as would be the case if the exposed gap junctional membranes were to provide a low resistance pathway to all small ions. The rapid healing-over of damaged areas in heart tissue is well known (Délèze, 1970; De Mello, 1972, 1975) and is promoted by extracellular Ca. Raising intracellular Ca can also produce cell uncoupling through changes in the electrical characteristics of gap junctional membranes (Rose, Simpson & Loewenstein, 1977; Loewenstein, Kanno & Socolar, 1978; see Coraboeuf, 1978, for other references)

including those in intercalated disks of Purkinje fibres (de Mello, 1975): intercalated disks of the isolated cells described here would obviously be exposed to 0·5–10·5 mM-$CaCl_2$ in the external solution. Both the healing-over process and reduction in the conductance of gap junctional membranes may facilitate viability in completely isolated heart cells through their actions on membrane structure. As pointed out by Carmeliet (1978), cell uncoupling would be a protective measure in the intact organ, since electrical insulation of cells in a damaged region of myocardium would reduce short-circuit currents to prevent depolarization and automatic activity.

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